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REVIEW

# Metals and amyloid-β in Alzheimer's disease

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## Summary

Mounting evidence is demonstrating roles for the amyloid precursor protein (APP) and its proteolytic product Aβ in metal homeostasis. Furthermore, aberrant metal homeostasis is observed in patients with Alzheimer's disease (AD), and this may contribute to AD pathogenesis, by enhancing the formation of reactive oxygen species and toxic Aβ oligomers and facilitating the formation of the hallmark amyloid deposits in AD brain. Indeed, zinc released from synaptic activity has been shown to induce parenchymal and cerebrovascular amyloid in transgenic mice. On the other hand, abnormal metabolism of APP and Aβ may impair brain metal homeostasis as part of the AD pathogenic process. Aβ and APP expression have both been shown to decrease brain copper (Cu) levels, whereas increasing brain Cu availability results in decreased levels of Aβ and amyloid plaque formation in transgenic mice. Lowering Cu concentrations can downregulate the transcription of APP, strengthening the hypothesis that APP and Aβ form part of the Cu homeostatic machinery in the brain. This is a complex pathway, and it appears that when the sensitive metal balance in the brain is sufficiently disrupted, it can lead to the self-perpetuating pathogenesis of AD. Clinical trials are currently studying agents that can remedy abnormal Aβ–metal interactions.

**Keywords**
Alzheimer's disease, amyloid-β peptide, amyloid precursor protein, metal homeostasis, copper, zinc, brain, transgenic mice

Alzheimer's disease (AD) is a polygenic neurodegenerative disorder involving the abnormal accumulation and deposition of a copper–zinc metalloprotein Aβ. Whether the aggregation of the peptide into amyloid deposits constitutes clearance of a toxic soluble entity or whether the amyloid plaques themselves are toxic is still debated. The aggregation of Aβ is mediated by interaction with metals, in particular zinc (Zn), copper (Cu) and iron (Fe) (Bush *et al*. 1994b; Atwood *et al*. 1998; Atwood *et al*. 2000b). Therefore, altered metal homeostasis may be an important factor leading to AD pathogenesis. Aβ also catalyses the reduction of $Cu^{2+}$ and $Fe^{3+}$ (Huang *et al*. 1999a), which in the absence of sufficient antioxidant mechanisms, could lead to the production of toxic reactive oxygen species (ROS) that may contribute to the pathogenesis of AD. Oxidative stress in turn may contribute to Aβ accumulation by generating modified Aβ species that have a high tendency to aggregate and are resistant to clearance (Kuo *et al*. 1998; Stadtman & Oliver 1991). Aβ is

generated by proteolytic cleavage of the amyloid precursor protein (APP). APP is processed by two competing pathways that either lead to (amyloidogenic pathway) or preclude (nonamyloidogenic pathway) Aβ production (Selkoe 1998). Aβ generation requires the consecutive action of two proteases, β- and γ-secretase, catalysing the release of the N- and C-termini of the Aβ molecule, respectively. The alternative α-cleavage of APP precludes Aβ formation by cleaving within the Aβ sequence. We review here the roles of metals in AD pathogenesis. In particular, we describe evidence for roles of APP and Aβ in Cu homeostasis, and how this may relate to the metal imbalances observed in AD brain, and the pathogenic process. The importance of brain metal homeostasis in Aβ accumulation and amyloid formation is also discussed.

## Metals and amyloid formation

Metals have been postulated to play a role in the pathogenesis of AD (Atwood *et al*. 1999; Bush 2000, 2003). Cu, Zn and Fe are concentrated in and around amyloid plaques in AD brain (Smith *et al*. 1997; Lovell *et al*. 1998; Sayre *et al*. 2000; Suh *et al*. 2000; Dong *et al*. 2003). High levels of Zn (Lee *et al*. 1999) and Fe (Smith *et al*. 1998a) have also been reported in the amyloid plaques of the Tg2576 (APPsw) mouse model for AD; however, to our knowledge, Cu levels have not yet been reported. Aβ possesses selective high and low affinity $Cu^{2+}$- and $Zn^{2+}$-binding sites that mediate its aggregation via interaction with $Cu^{2+}$, $Zn^{2+}$ and to a lesser extent $Fe^{3+}$ *in vitro* (Bush *et al*. 1994b; Atwood *et al*. 1998; Atwood *et al*. 2000b). Electron paramagnetic resonance and nuclear magnetic resonance studies proposed a model of monomer Aβ binding to a Cu ion via three histidines and a tyrosine or via a bridging histidine for aggregated Aβ (Curtain *et al*. 2001). Recently, Raman spectroscopic analysis of senile plaque cores demonstrated that Cu and Zn ions are co-ordinated via histidine residues (Dong *et al*. 2003), which are located at the N-terminal end of the Aβ sequence. The affinity of Aβ variants for $Cu^{2+}$ is greatest for Aβ1–42 > Aβ1–40 > > rat Aβ, correlating with redox activity and toxicity (Atwood *et al*. 1998; Huang *et al*. 1999b; Atwood *et al*. 2000b).

The exact role of metal ions in β-sheet formation and fibril assembly of Aβ peptides *in vivo* is unclear. Zinc is a more powerful inducer of Aβ aggregation than Cu or any other metal (Atwood *et al*. 1998; Atwood *et al*. 2000b). At neutral pH, $Zn^{2+}$ binds to Aβ to form insoluble aggregates, while $Cu^{2+}$ binding is competitive, inducing a soluble conformation (Clements *et al*. 1996; Miura *et al*. 2000). At mildly acidic pH, however, which occurs in an aged brain and in response to inflammation, $Cu^{2+}$ induces the formation of insoluble Aβ aggregates (Atwood *et al*. 1998). Indeed, altered co-ordination of Cu by the Aβ-histidine residues has recently been demonstrated with the lowering of pH (Syme *et al*. 2004).

The balance of Zn and Cu concentrations, as well as the maintenance of physiological pH, may therefore be important to prevent Aβ aggregation and amyloid formation. The dynamics of these metal ion interactions *in vivo,* however, are unknown. Chelation of metal ions reverses the aggregation of synthetic Aβ peptide and dissolves amyloid in postmortem human brain specimens (Huang *et al*. 1997; Atwood *et al*. 1998; Cherny *et al*. 1999). Furthermore, the treatment of the Tg2576 transgenic mouse model for AD with clioquinol, an orally bioavailable metal chelator, induced a marked inhibition of cortical amyloid accumulation (Cherny *et al*. 2001). This effect was recently reproduced using another hydrophobic chelator DP-109 (Lee *et al*. 2004a).

A recent study found that the formation of Aβ deposits in rabbits that resulted from a high cholesterol diet could be prevented by feeding demineralized water, instead of tap water, which contained traces of Cu (0.12 ppm) and presumably a mixture of other metals (Sparks & Schreurs 2003). This suggested that dietary metals could influence Aβ deposition in the brain under certain conditions. Perhaps in a brain, where Aβ clearance is already challenged by other factors (such as high cholesterol), dietary metals may further promote Aβ accumulation.

### *Synaptic Zn is important for amyloid-β formation*

A recent series of studies have demonstrated a key role for synaptic Zn in Aβ deposition and amyloid formation in Tg2576 mice (Lee *et al*. 2002; Friedlich *et al*. 2004; Lee *et al*. 2004b). Synaptic Zn is an exchangeable $Zn^{2+}$ pool in synaptic vesicles that is externalized upon neurotransmission, resulting in transient elevations of $Zn^{2+}$ in the extracellular spaces from a basal level of <0.5 μM [reviewed in (Bush 2000; Frederickson & Bush 2001)] to around 300 μM [reviewed in (Frederickson & Bush 2001)]. Mice deficient in synaptic Zn transporter (ZnT3) are deficient in synaptic Zn. Genetic ablation of ZnT3 in Tg2576 mice resulted in a approximately 50% reduction in amyloid burden compared with control Tg2576 mice (Lee *et al*. 2002). Interestingly, the highest levels of free or synaptic Zn are found in cortex and hippocampus, the regions most affected in AD (Frederickson *et al*. 1992; Bush 2003). $Zn^{2+}$ reuptake after synaptic release is a rapid, energy-dependent process. Hence, energy depletion could cause a pooling of extracellular $Zn^{2+}$, contributing to Aβ deposition (Bush 2003).

ZnT3 also regulates an exchangeable $Zn^{2+}$ pool, recently identified in the cerebrovascular wall of mice (Friedlich *et al*. 2004). Cerebral amyloid angiopathy (CAA) is common in AD

and may contribute to dementia and cerebral haemorrhage. The CAA in Tg2576 mice is enriched in this exchangeable $Zn^{2+}$ pool, and depletion of this $Zn^{2+}$ pool by genetic ablation of ZnT3 resulted in a dramatic reduction in CAA (Friedlich *et al*. 2004).

The ZnT3 regulatable synaptic Zn pool (which represents approximately 15% of total brain Zn) was shown to increase with age but only in female mice (Lee *et al*. 2002). Lee and co-workers subsequently demonstrated that synaptic Zn levels are regulated by oestrogen through the expression of an adaptor protein (Lee *et al*. 2004b). This age-dependent elevation in synaptic Zn accounted for the greater amyloid load in Tg2576 females, as genetic ablation of ZnT3 not only reduced the amyloid burden in both male and female Tg2576 mice but also removed the sex difference in amyloid load (Lee *et al*. 2002). Whether synaptic Zn also increases with age in humans is unknown. Furthermore, some (Sturchler-Pierrat & Staufenbiel 2000; Callahan *et al*. 2001; Wang *et al*. 2003) but not all (Oddo *et al*. 2003) transgenic mouse models of AD amyloidosis display enhanced pathology in females. This suggests that the age-dependent elevation in synaptic Zn may not be a phenomenon common to all mouse lines, or indeed across species.

Like Zn, Cu is also released with neurotransmission but at a concentration an order of magnitude less than Zn (Hartter & Barnea 1988a,b). Synaptic Cu could potentially participate in the formation of extracellular Aβ aggregates. However, far less is known about this release mechanism, and data on its role in experimental AD models are not yet available.

### *Age-dependent elevations in Aβ-precipitating metal ions*

Fe levels in the brain have been shown by several groups to rise markedly with age in both humans and mice (Drayer *et al*. 1986; Connor *et al*. 1992; Thomas *et al*. 1993; Bartzokis *et al*. 1997; Martin *et al*. 1998; Zecca *et al*. 2001; Maynard *et al*. 2002). Cu levels have also been shown to rise with age in mouse brain (Massie *et al*. 1979; Morita *et al*. 1994; Maynard *et al*. 2002), and therefore, probably also rise with age in human brain. Although the pools of Fe and Cu that increase with age have not been clearly defined, electron paramagnetic imaging has demonstrated clusters of both Cu and Fe ions in the brain that increase with age and are even further elevated in AD (Wender *et al*. 1992). This suggests the presence of redox-active metal ion stores with low bioavailability in the ageing brain. Together, a large body of data demonstrate an age-dependent breakdown of metal regulation that could be a common consequence of ageing. Increased levels of Cu, Fe and perhaps synaptic Zn levels may contribute to the age-dependent formation of amyloid pathology.

The literature available on the changes in total brain Zn levels with age is limited and inconsistent. However, the trend among reports on a range of mouse tissues including brain (Woodward *et al*. 1984; Morita *et al*. 1994) and human serum (Bohnen *et al*. 1994; Del Corso *et al*. 2000) is that Zn levels tend to either remain unchanged or show a slight decrease with age. Female mice have increasingly more exchangeable $Zn^{2+}$ in the ZnT3-associated cortical synaptic vesicles with advancing age (Lee *et al*. 2002), possibly contributing to increased amyloid plaque burden in APP transgenic mice.

## Metals and oxidative stress

Because of the redox-active nature of Cu and Fe, defective regulation of these metals can lead to reaction with $O_2$ and the production of ROS, resulting in cellular toxicity. AD brain exhibits marked oxidative damage of proteins, lipids and nucleic acids (Pappolla *et al*. 1992; Smith *et al*. 1994; Sayre *et al*. 1997; Hensley *et al*. 1998; Pratico *et al*. 1998; Smith *et al*. 1998b; Nunomura *et al*. 1999; Smith *et al*. 2000). Oxidative damage is highly concentrated in and around amyloid plaques, but immunohistochemical analysis of Tg2576 brain has shown markers of an oxidative stress response also in neuropil devoid of Aβ deposits (Pappolla *et al*. 1998; Smith *et al*. 1998a). Markers of lipid peroxidation are also found in cerebrospinal fluid (CSF) and urine of patients with a clinical diagnosis of AD (Pratico *et al*. 2000a; Tuppo *et al*. 2001), and levels increase with the progression of the disease. Young patients with Down's syndrome, where APP/Aβ is overexpressed, also display increased lipid peroxidation markers in urine compared with age-matched controls (Pratico *et al*. 2000b). Analysis of Tg2576 mice, which display oxidative damage similar to that found in AD brain (Smith *et al*. 1998a), revealed an elevation in oxidative stress markers preceding amyloid formation and increasing with the age-dependent development of amyloid pathology (Pratico *et al*. 2001). Together, data from humans and transgenic mice indicate that elevated oxidative stress is an early event in AD pathogenesis and may therefore contribute to the range of pathological changes observed.

The production of oxygen radicals is a normal consequence of metabolic activity, but cellular antioxidant defences such as cytosolic Cu/Zn superoxide dismutase (SOD1), catalase, glutathione peroxidase, haemoxygenase and mitochondrial manganese (Mn) superoxide dismutase (SOD2), along with other nonenzymatic antioxidants, defend against oxidative damage [reviewed in (Perry *et al*. 2002b)]. The brain is particularly vulnerable to oxidative stress because of its high metabolic rate, utilizing 20% of basal oxygen consumption. In addition, the brain has limited antioxidant defences compared with

other organs (Floyd 1999) and high levels of transition metals, which can generate oxygen radicals.

## Metals and Aβ toxicity

Aβ is central to the pathogenesis of AD. The Aβ peptide is toxic to neurones in cell culture, and this toxicity has been shown to be mediated by the interaction of the peptide with $Cu^{2+}$ and $Fe^{3+}$ (Huang *et al.* 1999b; Rottkamp *et al.* 2001; Opazo *et al.* 2002). Aβ catalyses the reduction of $Cu^{2+}$ to $Cu^{+}$ and $Fe^{3+}$ to $Fe^{2+}$, generating $H_2O_2$ from molecular oxygen ($O_2$) and available biological reducing agents such as vitamin C, cholesterol and catecholamines (Opazo *et al.* 2002). In the absence of sufficient detoxifying enzymes such as catalase and glutathione peroxidase, $H_2O_2$ will further react with reduced metal ions such as $Fe^{2+}$ and $Cu^{+}$ to generate toxic hydroxyl radicals (Fenton reaction). The toxicity of Aβ42 is greater than Aβ40 (Huang *et al.* 1999b; Cuajungco *et al.* 2000; Rottkamp *et al.* 2001) correlating with their relative $Cu^{2+}$- and $Fe^{3+}$-reducing potentials and the ability to catalytically generate $H_2O_2$ from biological reducing agents (Huang *et al.* 1999a; Opazo *et al.* 2002). Interestingly, murine $APP^{-/-}$ neurones are less susceptible to Cu-induced toxicity than wild-type neurones, whereas knockouts of APLP2 (which shares a homologous N-terminal metal-binding domain with APP but does not produce Aβ) are not protected against Cu-induced toxicity (White *et al.* 1999a).

The formation of amyloid deposits *per se* does not correlate with the clinical severity of AD. Instead, levels of soluble Aβ were found to correlate with the severity of the clinical symptoms of AD (Lue *et al.* 1999; McLean *et al.* 1999; Wang *et al.* 1999). AD brain has elevated levels of soluble Aβ as well as aggregated Aβ. Recent evidence suggests that soluble dimeric and oligomeric forms of Aβ are more toxic in cell culture than monomeric Aβ (Dahlgren *et al.* 2002; Walsh *et al.* 2002a) and may mediate toxicity in AD (Mucke *et al.* 2000; Walsh *et al.* 2002b).

Soluble Aβ oligomers that generate ROS by interaction with Cu and Fe may be a transient species that precede the formation of relatively inert amyloid complexes. A number of stress conditions upregulate APP expression and amyloidogenic processing of APP to generate Aβ (Misonou *et al.* 2000; Paola *et al.* 2000; Cheng & Trombetta 2004) [reviewed in detail in (Atwood *et al.* 2003)]. Evidence that Aβ can act as an antioxidant under certain conditions [summarized in (Atwood *et al.* 2003)] suggests that Aβ production, in conjunction with its neuroprotective and neurotrophic properties (Whitson *et al.* 1989; Whitson *et al.* 1990; Yankner *et al.* 1990; Koo *et al.* 1993; Luo *et al.* 1996; Chan *et al.* 1999), may be a stress response to minimize oxidative damage. If small Aβ–metal aggregates and oligomers that are formed during such stress responses are not rapidly degraded, they may cause further damage. With the further recruitment of metals, these oligomeric and aggregated Aβ species may become subsequently sequestered into amyloid which is less toxic than soluble Aβ oligomers.

The elevated oxidative stress levels in AD brain may operate in tandem with abnormalities in tissue Cu and Fe to engender Aβ oligomer formation and amyloid deposition. An oxidative environment has been demonstrated to result in the formation of a di-tyrosine linkage between two Aβ peptides (Galeazzi *et al.* 1999), hence promoting the irreversible oligomerization of Aβ peptides. $Cu^{2+}$ directly interacts with Aβ to foster di-tyrosine cross-linking and covalent oligomerization (Atwood *et al.* 2004; Barnham *et al.* 2004). AD brain indeed possesses a high content of di-tyrosine (Atwood *et al.* 2000a). Oxidative reactions may also facilitate the accumulation of Aβ via the formation of covalent cross-links between Aβ peptides (Atwood *et al.* 2000a; Atwood *et al.* 2000b; Loske *et al.* 2000; Palmblad *et al.* 2002) and between other proteins [discussed in (Perry *et al.* 2002a)], generating larger protein aggregates that resist clearance (Stadtman & Oliver 1991; Friguet *et al.* 1994; Kuo *et al.* 1998). Interestingly, the oxidative modification of the sulphur atom of Met35 of Aβ by reaction with $Cu^{2+}$ has been shown to reduce the affinity of Aβ for membranes (Barnham *et al.* 2003a). Oxidative modifications of Aβ may therefore also contribute to the increased levels of toxic, soluble Aβ oligomers, causing toxicity and neurodegeneration in AD, prior to the peptides becoming incorporated into relatively inert amyloid complexes.

The role of $Zn^{2+}$ in Aβ toxicity is complex. *In vitro*, $Zn^{2+}$ inhibits Aβ toxicity in cell cultures (Lovell *et al.* 1999; Cuajungco *et al.* 2000), possibly by quenching $H_2O_2$ production from Aβ:$Cu^{2+}$ complexes (Cuajungco *et al.* 2000). As $Zn^{2+}$ forms plaques *in vivo*, the quenching of Aβ redox activity is consistent with the observation that oxidation damage in the brain in AD inversely correlates with the plaque burden (Cuajungco *et al.* 2000). However, even in the most heavily plaque-burdened brains, there is still marked oxidation damage, which may be mediated by soluble or diffuse forms of Aβ (Cuajungco *et al.* 2000).

## Abnormal metal homeostasis in AD

Numerous reports have demonstrated transition metal imbalances in AD brain, such as increased Fe, Zn and Mn (Samudralwar *et al.* 1995; Deibel *et al.* 1996; Danscher *et al.* 1997; Cornett *et al.* 1998; Rao *et al.* 1999), and importantly, decreased Cu (Deibel *et al.* 1996; Plantin *et al.* 1987; Loeffler *et al.* 1996; Rao *et al.* 1999). CSF and serum of AD patients

tend to show the opposite trend to brain, with increased serum and CSF Cu levels (Basun *et al*. 1991; Gonzalez *et al*. 1999; Squitti *et al*. 2002) and decreased serum Fe and Zn (Loeffler *et al*. 1994; Molina *et al*. 1998), suggesting a redistribution of metals between the brain and the fluids responsible for supplying and clearing excreted metals from the brain.

Imbalances in metal levels in the AD brain may reflect deficiencies or excesses of particular metalloproteins or defective metal transporters. Indeed, the levels of several Cu and Fe regulatory and storage proteins are altered in AD brain (Basun *et al*. 1991; Connor *et al*. 1993; Loeffler *et al*. 1994; Loeffler *et al*. 1996; Smith *et al*. 1998c; Castellani *et al*. 1999) as are several essential Cu-dependent enzymes (see next section). Iron and Cu form essential components of several enzymes required for vital brain functions including energy production, neurotransmitter synthesis and antioxidant function.

### *AD brain is deficient in bioavailable copper and cuproenzymes*

Several important enzymes that are altered in AD brain require Cu for their catalytic activity. Cytochrome C-oxidase (COX), a Cu-dependent enzyme involved in mitochondrial respiration, is essential for energy production in the brain. Several studies have shown decreased COX levels (Cottrell *et al*. 2001) or activity (Maurer *et al*. 2000) and defective energy metabolism (Duara *et al*. 1986; McGeer *et al*. 1990; Mielke *et al*. 1992; Mielke *et al*. 1996) in AD brain. In fact, inherited mutations in mitochondrial COX genes have been shown to segregate with greater frequency with late onset AD cases (Davis *et al*. 1997). This supports defective COX activity as a risk factor for AD. The APP mismetabolism that occurs in AD may impact upon the ability of COX to obtain adequate copper.

Ceruloplasmin comprises a major reservoir of the body's copper. Ceruloplasmin levels in AD patients have also been found to be decreased in the brain tissue (Connor *et al*. 1993) and increased in the CSF (Basun *et al*. 1991; Loeffler *et al*. 1994). Ceruloplasmin is a key regulator of Fe transport and is important for the inhibition of Fe-induced lipid peroxidation of proteins by oxidizing $Fe^{2+}$ to $Fe^{3+}$ leading to the incorporation of Fe into ferritin (Samokyszyn *et al*. 1991; de Silva & Aust 1993).

The activity of peptidylglycine α-amidating monooxygenase (PAM), a Cu-dependent enzyme involved in neuropeptide and peptide hormone processing, has been shown to decrease by 16% per year in the CSF of AD patients, and lower PAM activity levels are also found in temporal lobe of AD brain at postmortem (Wand *et al*. 1987). The possibility that these decreases are due to a broad cell loss is negated by the findings that other markers, such as oxidative stress-handling enzymes (SOD1, HO-1, catalase, glutathione peroxidase and glutathione reductase) are elevated in AD brain (Pappolla *et al*. 1992; Aksenov *et al*. 1998; Omar *et al*. 1999).

Expression of the highly abundant antioxidant enzyme Cu/Zn-superoxide dismutase (SOD1) has been shown to be elevated in AD brain (Omar *et al*. 1999) and in transgenic APP mice (Bayer *et al*. 2003). However, in both cases, SOD1 activity is decreased, suggesting that the decreased SOD1 activity is due to a reduction in the active site Cu occupancy. Interestingly, dietary supplementation with Cu was found to restore SOD1 activity in transgenic APP mice and improve survival of the mice (Bayer *et al*. 2003). This suggests that Cu deficiency in AD may play a role in the disease development.

The mechanism of the Cu depletion and the pools of Cu affected in AD remain largely unknown. Free Cu is not normally found *in vivo* (Rae et al. 1999), as its highly reactive nature can participate in oxygen radical formation via Fenton and Haber-Weiss reactions. Cu is delivered to its target proteins, transported across cell membranes and between cellular compartments by specific Cu chaperones [reviewed in (Shim & Harris 2003)]. Although several Cu chaperones have been identified, much remains to be known about the proteins and mechanisms involved in Cu transport and regulation, particularly in the brain.

## Roles of APP and Aβ in metal homeostasis

### *APP and Aβ impact upon metal homeostasis: a possible metal transport mechanism*

The high energy, positively cooperative hexameric structure adopted by Aβ when binding $Cu^{2+}$ and $Zn^{2+}$ (Curtain *et al*. 2001; Curtain *et al*. 2003) implies that the complex subserves a possible physiological function. There is an interdependent relationship between Cu levels, APP expression and Aβ production supporting a role for APP and Aβ in Cu efflux. The initial finding showed that the APP and amyloid precursor-like protein 2 (APLP2) knockout mice have specific elevations in brain and liver Cu levels (White *et al*. 1999b). APLP2 shares a homologous N-terminal Cu-binding domain with APP but does not produce Aβ (Bush *et al*. 1993; Hesse *et al*. 1994). Primary cortical neurones and embryonic fibroblasts from APP and APLP2 double knockout mice also display significantly elevated Cu levels (Bellingham *et al*. 2004a). Conversely, overexpression of APP has been consistently shown to result in decreased Cu levels in three independent transgenic mouse lines (Maynard *et al*. 2002; Bayer *et al*. 2003; Phinney *et al*. 2003) and in primary cortical neurones (Bellingham *et al*. 2004a).

Overexpression of APP-C100, which contains Aβ but not the N-terminal $Zn^{2+}$-and $Cu^{2+}$-binding domain of APP, results in decreased brain Cu levels as well as Fe levels (Maynard *et al.* 2002). This suggests a role for Aβ in both Cu and Fe homeostasis. The effect of Aβ on Fe levels supports the previous proposal that the APP/Aβ system may play a role in the removal of excess Fe from the cell (Bush 2003). This stems from the identification of an iron-regulatory element (IRE-Type II) in the 5′-untranslated region of APP (Rogers *et al.* 2002). Alternatively, the decrease in Fe may reflect a homeostatic adjustment to the reduction in Cu levels.

Although Cu is the trace metal most strikingly affected in these transgenic and knockout mouse models, other less pronounced metal level changes may have some importance. The Cu deficit in Tg2576 mice was accompanied by a small but significant decrease in Zn levels (Maynard *et al.* 2002), whereas the Cu increases in $APP^{-/-}$ mice were accompanied by nonsignificant increases in Zn (White *et al.* 1999b). Interestingly, in both studies, the molar Zn changes were of similar magnitude to the molar Cu changes. However, because of the greater abundance of Zn in the brain, these changes corresponded to a smaller percentage of total Zn than Cu. The effects on Zn could therefore be a direct result of APP/Aβ expression.

Both APPsw (Tg2576) and APP-C100 mice displayed significant elevations in Mn levels (Maynard *et al.* 2002). Because Mn does not appreciably interact with Aβ or APP (Bush *et al.* 1993; Bush *et al.* 1994a; Bush *et al.* 1994b; Atwood *et al.* 1998), the increased Mn levels are more likely a secondary effect of altered metal homeostasis. Elevated Mn levels have also been found in AD brain (Rao *et al.* 1999). Most Mn in the brain is bound to metalloproteins such as glutamine synthetase and mitochondrial Mn superoxide dismutase (SOD2). A portion of Mn also exists in the synaptic vesicles of glutamatergic neurones and is released into the synaptic cleft along with glutamate, participating in the regulation of synaptic neurotransmission (Takeda 2003).

Abnormally high concentrations of brain Mn have been shown to cause an irreversible neurological syndrome similar to Parkinson's disease (Aschner 1997), hence, the elevated Mn in AD brain may contribute to the pathology. Decreased Cu and increased Mn levels have been observed in other neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and Creutzfeldt–Jakob disease (CJD) (Kapaki *et al.* 1997; Wong *et al.* 2001). Brain tissue from CJD patients displays decreased Cu and increased Mn levels. Furthermore, prion protein extracted from CJD brain displays a decreased Cu and increased Mn contents (Wong *et al.* 2001). Interestingly, replacement of Cu by Mn facilitates misfolding of the prion protein in favour of higher β-sheet content, protease resistance and loss of antioxidant function (Brown *et al.* 2000). However, Mn-induced aggregation of Aβ has not been demonstrated by *in vitro* studies using synthetic peptides (Bush *et al.* 1994b; Atwood *et al.* 1998). Decreased Cu and increased Mn levels have also been found in the serum and CSF (Mn increase nonsignificantly) of ALS patients (Kapaki *et al.* 1997). Although the relationship between Cu and Mn changes is not well understood, opposing changes in the levels of Cu and Mn are emerging as a common trait of neurological diseases.

The Cu-binding domain of APP shows structural homology to Cu chaperones (Barnham *et al.* 2003b), supporting the notion that the APP (and APLP2) Cu-binding domain functions as a neuronal metal transporter and/or chaperone to modulate Cu homeostasis. Furthermore, APP gene expression has recently been shown to be downregulated by Cu depletion (Bellingham *et al.* 2004b), suggesting a negative feedback mechanism evolved to preserve intracellular Cu levels. The Cu stores depleted by APP and Aβ overexpression remain unknown, as is the mechanism by which this occurs.

The main problem with distinguishing whether the effects on Cu homeostasis are elicited by the APP N-terminal Cu-binding domain or Aβ, both of which bind and catalyse the reduction of $Cu^{2+}$, is that most of the models studied to date utilize the overexpression or ablation of APP, resulting in the joint alterations in the levels of both APP and Aβ. The only direct evidence for Aβ causing Cu efflux, independently of APP, is that Cu levels are decreased in mice overexpressing APP-C100 (Maynard *et al.* 2002). Furthermore, the magnitude of the Cu elevation in $APP^{-/-}$ mice is greater than that in $APLP2^{-/-}$ mice (White *et al.* 1999b), suggesting that the increased Cu may be due to the joint effects of the APP N-terminal Cu-binding domain and Aβ rather than the action of the N-terminal copper-binding domain alone, because this domain is homologous in both APP and APLP2. No consensus has been reached as to whether APP expression is altered in AD, except in cases resulting from Down's syndrome or head trauma. The majority of genetic and biochemical evidence links increased the production of Aβ or Aβ42 with AD, rather than increased APP expression. Hence, APP-overexpressing models may not be an accurate reflection of the Cu depletion observed in AD. These models do however increase our understanding of the roles of APP and Aβ in metal homeostasis.

### *Cu promotes the nonamyloidogenic APP processing pathway*

An important link between Cu levels and amyloid formation has recently been unveiled by two independent and complementary studies using two different transgenic APP mouse models. Both studies reported decreased constitutive brain

Cu levels (Bayer *et al.* 2003; Phinney *et al.* 2003) in agreement with earlier findings (Maynard *et al.* 2002). Elevation of brain Cu levels, either by dietary Cu supplementation (Bayer *et al.* 2003) or by the introduction of a mutant allele of the CuATPase7b Cu transporter (Phinney *et al.* 2003), improved the survival of the mice and resulted in a marked decrease in Aβ and amyloid plaque load. These findings suggest that elevated Cu may drive nonamyloidogenic processing of APP, as demonstrated previously *in vitro* (Borchardt *et al.* 1999).

This is important, as it suggests that the decreased Cu levels in AD brain could further increase Aβ production, perpetuating a pathogenic cascade of events. However, if low Cu availability in the brain signals the downregulation of APP expression as occurs *in vitro* (Bellingham *et al.* 2004b), this may provide a protective mechanism to limit the amount of Aβ production in an environment where amyloidogenic APP processing is favoured. However, in the transgenic mouse models, which use non-APP promotors, this protective mechanism is absent. Hence, Cu supplementation in humans may not be expected to exert such a profound effect on Aβ levels and amyloid formation. Replenishment of deficient Cu stores in humans may decrease the proportion of APP that undergoes amyloidogenic processing; however, concurrent upregulation of APP expression may further add to the net Aβ burden (Figure 1).

When attempting to predict the effects of Cu supplementation on human AD patients, the potential benefits of restoring the apparent Cu deficiency on enzyme activities and perhaps nonamyloidogenic processing of APP must be weighed against the potential risk of exacerbating the condition by increasing the availability of Cu for the formation of toxic Aβ oligomers and the generation of ROS, as well as possibly increasing the APP expression. The correlation of the findings obtained from mice to humans is limited not only by the non-native regulation of transgenic APP expression and the lack of the full spectrum of AD pathology in transgenic APP mice but also by the differences between humans and mice in their metal-regulatory machinery.

A recent clinical trial of the Cu/Zn chelator clioquinol has revealed promising effects, with a modest slowing of cognitive decline and a parallel decrease in serum Aβ42 (Ritchie *et al.* 2003). The effects of oral clioquinol treatment on Tg2576 mice were a striking reduction in brain Aβ levels and amyloid plaque load (Cherny *et al.* 2001), as has been since achieved with another hydrophobic chelator DP-109 (Lee *et al.* 2004a). Although clioquinol treatment results in decreased Cu, Fe and cobalt levels in nontransgenic mice (Yassin *et al.* 2000), Tg2576 mice treated with clioquinol displayed paradoxical elevations in brain Cu and Zn levels (Cherny *et al.* 2001). A plausible explanation for the findings is that clioquinol

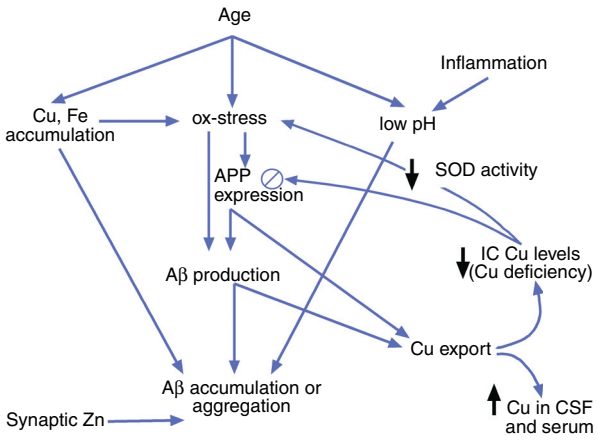


**Figure 1** Proposed mechanism for copper (Cu) depletion and β-amyloid formation in humans. Increasing age leads to the accumulation of Cu and iron (Fe) deposits, elevated oxidative stress and reduced pH in the brain, all of which may promote the aggregation of Aβ. Oxidative stress and other metabolic stresses may also promote Aβ production by jointly upregulating amyloid precursor protein (APP) expression and driving amyloidogenic processing of APP (Misonou *et al.* 2000; Paola *et al.* 2000; Atwood *et al.* 2003; Cheng & Trombetta 2004). Higher APP expression and elevated Aβ levels cause greater than required Cu export, leading to increased Cu in cerebrospinal fluid (CSF) and serum, and an intracellular (IC) Cu deficiency in the brain. Cu-deficient superoxide dismutase (SOD1) contributes to the reduced antioxidant capacity of the brain, allowing further oxidative stress. Unlike in transgenic mouse models, decreased intracellular Cu levels may downregulate APP expression (Bellingham *et al.* 2004a) in an attempt to conserve Cu levels. In Alzheimer's disease (AD) brain, this negative feedback must be insufficient to counteract other factors that promote APP expression, Aβ production and Aβ accumulation. Consequently, a Cu deficiency develops along with the classic Aβ-amyloid pathology of AD. Zn – zinc.

treatment loosens amyloid plaques and aggregates and solubilizes smaller Aβ oligomers that would otherwise exert toxicity and become sequestered into amyloid deposits. The liberation of Aβ into more soluble forms allows more efficient clearance of Aβ. Hence, with prolonged treatment, much of the existing amyloid burden has been cleared, and the continued growth of amyloid deposits is prevented. The observation that Cu and Zn levels were elevated suggests that by achieving lower total Aβ levels, the excessive Cu export that occurs in Tg2576 mice is attenuated. The elevation in Zn levels, however, cannot be explained by the reduction in Aβ levels, as APP-C100 mice do not display decreased Zn levels (Maynard *et al.* 2002). The Cu and Zn elevations could be due in part to Cu and Zn ions liberated from amyloid plaques that are still bound to clioquinol in the brain. However, the net increase in Cu and Zn levels in the brain in the presence of fewer amyloid

deposits indicates either greater uptake or reduced export. Hence, the Cu and Zn elevations may be a result of favourable changes to metal homeostasis resulting from the clioquinol treatment. Approximately, 15% of plasma Zn in mice is in communication with Zn released in cortical synapses associated with ZnT3 activity (Friedlich *et al.* 2004). The normalization of plasma zinc (rising from below normal baseline levels) as a result of clioquinol treatment in AD patients might be explained by the dissolution of parenchymal and cerebrovascular amyloid permitting the re-establishment of communication between plasma and synaptic Zn (Ritchie *et al.* 2003).

## Concluding remarks

Experimental evidence from transgenic mouse models that the APP/Aβ system forms part of the brain's Cu-regulatory machinery has revealed the possibility that the corruption of Aβ metabolism in AD brain may not only result in an intracellular Cu deficiency, but this Cu deficiency may further propel amyloidogenesis. Future research will elucidate the mechanisms by which APP and Aβ regulate Cu transport and the pools of Cu and other metal ions that are affected. These findings will help predict the efficacy of therapeutic strategies targeting metals in the brain, such as chelation therapy or metal supplementation.

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